



## Research article

# An inhibitory pathway controlling the gating mechanism of the mouse lateral amygdala revealed by voltage-sensitive dye imaging



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## HIGHLIGHTS

- EC stimulation induces large and long-lasting hyperpolarizing signals in the La.
- This hyperpolarization is analyzed by VSD imaging spatially and temporally.
- We identify an inhibitory pathway toward the La via the m-ITC.

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## ABSTRACT

The lateral amygdala nucleus (La) is known as a gateway for emotional learning that interfaces sensory inputs from the cortex and the thalamus. In the La, inhibitory GABAergic inputs control the strength of sensory inputs and interfere with the initial step of the acquisition of fear memory. In the present study, we investigated the spatial and temporal patterns of the inhibitory responses in mouse La using voltage-sensitive dye imaging. Stimulating the external capsule (EC) induced large and long-lasting hyperpolarizing signals in the La. We focused on these hyperpolarizing signals, revealing the origins of the inhibitory inputs by means of surgical cuts on the possible afferent pathways with four patterns. Isolating the medial branch of EC (ECmed), but not the lateral branch of EC (EClat), from the La strongly suppressed the induction of the hyperpolarization. Interestingly, isolating the ECmed from the caudate putamen did not suppress the hyperpolarization, while the surgical cut of the ECmed fiber tract moderately suppressed it. Glutamatergic antagonists completely suppressed the hyperpolarizing signals induced by the stimulation of EC. When directly stimulating the dorsal, middle or ventral part of the ECmed fiber tract in the presence of glutamatergic antagonists, only the stimulation in the middle part of the ECmed caused hyperpolarization. These data indicate that the GABAergic neurons in the medial intercalated cluster (m-ITC), which receive glutamatergic excitatory input from the ECmed fiber tract, send inhibitory afferents to the La. This pathway might have inhibitory effects on the acquisition of fear memory.

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## 1. Introduction

The amygdala is an important brain structure for emotional behavior and learning [13]. Fear conditioning is a widely-used experimental model to examine emotional and learning processing in animal brains. The lateral amygdala nucleus (La) is known as a

gateway for emotional learning that interfaces sensory inputs from the cortex and the thalamus [14]. Inhibitory circuits are known to control the amygdala's functions, such as acquisition, expression, and extinction of conditioned fear [6,21,22]. Inhibitory inputs to the La control the strength of sensory inputs and interfere with the initial step of the acquisition of fear memory. Two groups of GABAergic neurons in the amygdala are known: local interneurons that are scattered within the local neuropil [17], and intercalated cells organized in clusters (intercalated clusters) surrounding the amygdala complex [15,16,18,20,23,24]. Although inhibitory inputs to the individual principal neurons in the amygdala have been analyzed electrophysiologically [4,26,31,37,38], how sensory inputs

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induce inhibitory responses in the La, and how inhibitory responses propagate in the La, are still unclear because of the technical limitations of patch clamp recording.

Optical imaging techniques overcome these limitations to investigating propagations in a wide range of neuronal interactions, and have been applied in the study of excitatory circuits of several brain regions [7,9,11,12,29,33,34]. In this study, using optical imaging techniques, we investigate neuronal activities in the La, focusing particularly on inhibitory responses. To identify the origins of the inhibitory inputs, we perform various patterns of knife-cut operations of the possible afferent pathways evoking hyperpolarization in the La. In addition, we investigate the effects of glutamatergic antagonists on the inhibitory responses in the La, and show an inhibitory pathway from the medial intercalated cluster (m-ITC) to the La.

## 2. Materials and methods

### 2.1. Slice preparation and staining procedure

The experimental protocol was reviewed and approved by the National Institute of Health Sciences (NIHS) in Japan, following the guidelines in the National Research Council's 'Guide for the Care and Use of Laboratory Animals'. All experiments were approved by the NIHS' ethics committee. Male mice (C57BL/6J, 7–22 weeks old, Japan SLC, Inc., Japan) were deeply anesthetized with halothane and quickly decapitated. Coronal slices containing the amygdala complex (400  $\mu$ m) were prepared using a vibrating microtome (Campden Instruments Ltd., Loughborough, UK) in ice-cold artificial cerebrospinal fluid (ACSF). The ACSF was composed of the following (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO<sub>4</sub>, 2.5 CaCl<sub>2</sub>, 1.0 NaH<sub>2</sub>PO<sub>4</sub>, 26.2 NaHCO<sub>3</sub>, and 11.0 glucose; this was oxygenated with a mixture gas of 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4). The slices were immediately soaked in the oxygenated ACSF containing a voltage-sensitive dye (VSD), di-4-ANEPPS (50  $\mu$ M, Invitrogen Molecular Probes Inc., Oregon, USA) for 10 s, and then transferred to a filter that absorbed the staining solution and subsequently to another filter that absorbed the normal ACSF for at least an hour before the experiment.

### 2.2. Experimental apparatus for VSD imaging

An epi-illumination macro zoom fluorescence microscopy (MVX-10 MacroView, Olympus, Japan), a LED light source with a 530 nm center wavelength (LEX2-Green, Brainvision Inc., Tokyo, Japan), a dichroic mirror (560 nm), an emission filter (BP 575–625 nm), and a CMOS imaging device (MiCAM ULTIMA-L, Brainvision Inc., Tokyo) were used for VSD imaging.

The decrease and increase in the fluorescent intensity from the preparation corresponded to the membrane depolarization and hyperpolarization, respectively. Each data acquisition consisted of 1024 images of consecutive frames (1.0 ms/frame). A coaxial needle electrode (TF203-047, Unique Medical Co. Ltd., Tokyo, Japan) was placed on the external capsule (EC). Electrical stimuli with 200- $\mu$ s duration at various intensities from 15 to 90  $\mu$ A were delivered at the 100th frame of each acquisition. To analyze the effects of deafferentation on the induction of the hyperpolarization, the stimulus intensity was adjusted to make the peak value of depolarization equal before and after the surgical cut. Methods to calculate optical signals and present images were described in previous papers [11,29,32].

### 2.3. Surgical cuts of afferent connections to the La

After recording the optical signals at various stimulation intensities, we performed knife-cut operations on the pathways assumed to be involved in the induction of the hyperpolarization in the La.

Four afferent pathways to the La were cut under the macro scope observation, as follows: the La was isolated by the longitudinal cut from: (i) the lateral branch of the EC, (ii) the medial branch of the EC and (iii) the CPu, and by (iv) the transverse cut of ECmed at the dorsal part. For the sham operations, the slices remained intact but the same procedure was carried out. After the surgical cuts, the slices were stored in the recovery chamber at room temperature (at least 1 h).

### 2.4. Excitation and inhibition values

Images from the 251st–300th frames were stacked and averaged to determine regions of interest (ROIs), which were circles of 8 pixels in diameter. Two ROIs were defined for each experiment. One of the ROIs had the maximal hyperpolarization value in the center spot. The other was adjacent to the first, which had an adequately large hyperpolarization value within the region. For the after-operation analysis, the ROIs were centered on the spots that had the same distances from the position of the stimulating electrode and the EC as the before-operation analysis. The averaged optical signals of the two ROIs were used as representative data.

The excitation (*E*) value was determined as the largest value among all the values from the first to the 15th frame after electrical stimulation. The inhibition (*I*) value was determined by averaging 50 frames, from the 251st frame to the 300th frame after the electrical stimulation.

### 2.5. Statistical analysis

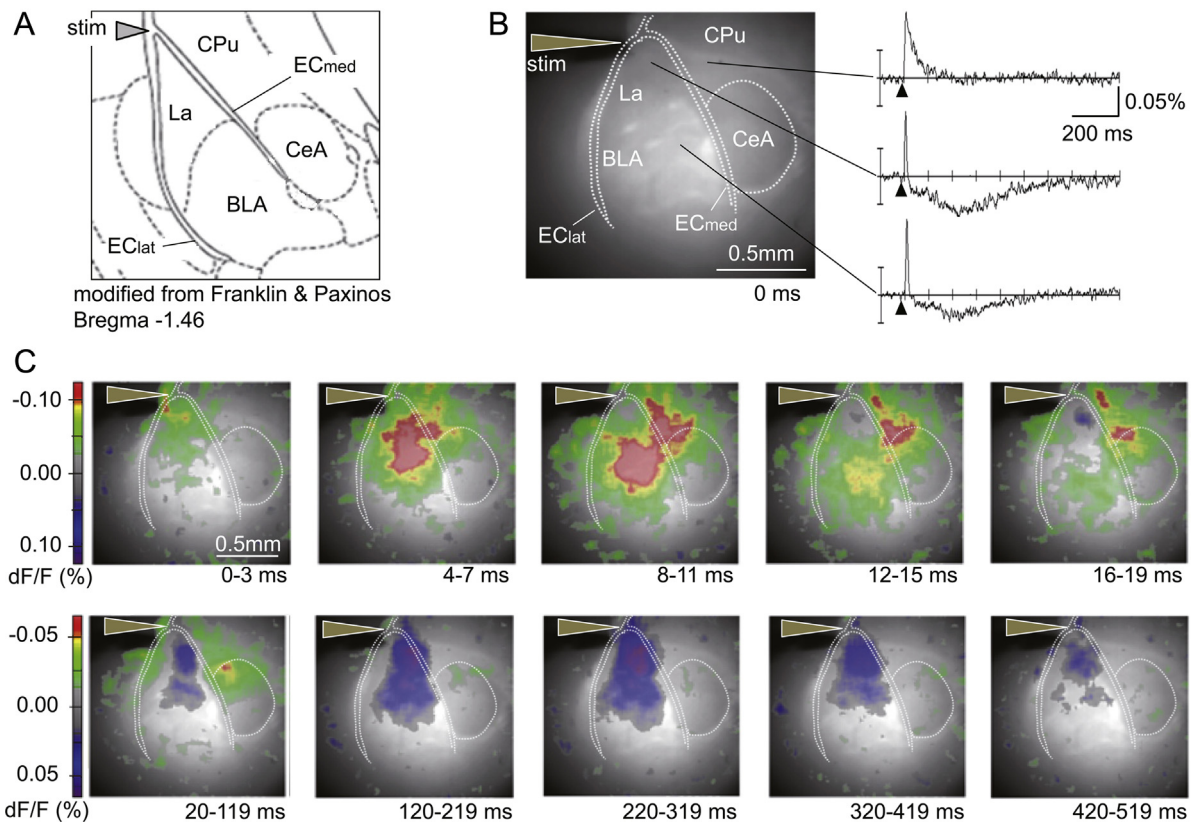
We defined the operation index (OI) as follows:  $OI = [I/E]_a/[I/E]_b$ , where a: after the operation, b: before the operation. The data were presented as mean  $\pm$  standard error of the mean (SEM). Normality of the data was tested with the Shapiro–Wilk test, and subsequently one-way ANOVA followed by Dunnett's post-hoc test was carried out. In the pharmacological experiments, inhibition value was statistically analyzed using a paired *t*-test.

## 3. Results

### 3.1. Optical signal propagation after the EC stimulation

The anatomical nomenclatures related to our experiments are shown in Fig. 1A. Each white fiber bundle in the amygdala slice preparation was observed through the macro zoom microscope; thus, the ramifying point of the EC was identified. The La, BLA, and CeA of the amygdala complex were identified in the fluorescent image recorded by the system (Fig. 1B). In Fig. 1C, a typical example of the optical signal propagation is shown in pseudo-color representation. The depolarization started at the stimulation point and spread over the La within 3 ms after the stimulation (0–3 ms). It became stronger in the La and spread over the other regions, the BLA, CPu, and CeA (4–7 ms). Then the depolarization at the dorsal area of the La faded, while growing stronger in the other regions (8–11 ms). Following depolarization, a weak hyperpolarization was first observed in the La; the depolarization remained in the other regions, although it was weakened (12–15 ms). The hyperpolarization grew stronger, and the depolarization in the other regions gradually disappeared (16–19 ms). The hyperpolarization in the La lasted about 600–650 ms (the middle wave in Fig. 1B). The maximal value of the hyperpolarization was observed around 255 ms after the stimulation.

After the hyperpolarization started in the La at the area along the dorsal part of the ECmed (16–19 ms), it spread out in the La during the next 100 ms (20–119 ms), then it spread over the BLA and a narrow part of the CPu along the dorsal part of the ECmed



**Fig. 1.** Optical signal propagation after the EC stimulation.

(A) The modified schematic diagram from the atlas of Franklin and Paxinos (2007) at Bregma -1.46 mm. The arrowhead shows the ramifying point of the external capsule (EC); lateral branch of the external capsule (EClat) and medial branch of the external capsule (ECmed). La – lateral amygdala nucleus; BLA – basolateral amygdala nucleus; CeA – central amygdala nucleus; CPu – caudate putamen. (B) Left, the fluorescence image of VSD-stained coronal slice. Right, a typical example of optical signals in the CPu (in the top wave), La (in the middle wave) and BLA (in the bottom wave). Brown arrowhead, the stimulating site; black arrowhead, the timing of electrical stimulation. (C) Images of optical signal changes produced at the various indicated times after electrical stimulation. In the upper panels, images for 4 frames were stacked to show the spread pattern of depolarization. In the lower panels, images for 100 frames were stacked to show the spread pattern of hyperpolarization.

during the following 100 ms (120–219 ms). The maximal hyperpolarization was observed in the La along the dorsal part of the ECmed from 220 to 319 ms. The strong and long-lasting hyperpolarization was mainly observed in the La (the middle wave in Fig. 1B), while the hyperpolarization in the BLA was weak (the bottom wave). The hyperpolarization was neither observed in the main part of the CPu (the top wave) nor in the CeA, while the depolarization in the CPu lasted longer than in the La and the BLA.

### 3.2. Effects of the deafferentation on the optical signals

To reveal the afferent pathway responsible for evoking the strong and long-lasting hyperpolarization, effects of the various surgical operations were investigated.

Deafferentation from the EClat to the La did not affect the induction of the strong and long-lasting hyperpolarization in the La (Fig. 2A), and there was no significant change in the OI value compared with the sham (Fig. 2E), suggesting that the afferent inputs via the EClat are not involved in the hyperpolarization induced in the La. In contrast, deafferentation from the ECmed to the La strongly reduced the hyperpolarization (Fig. 2B). OI values were also significantly reduced (Fig. 2E). These results suggest that the inputs via the ECmed are involved in the hyperpolarization induced in the La.

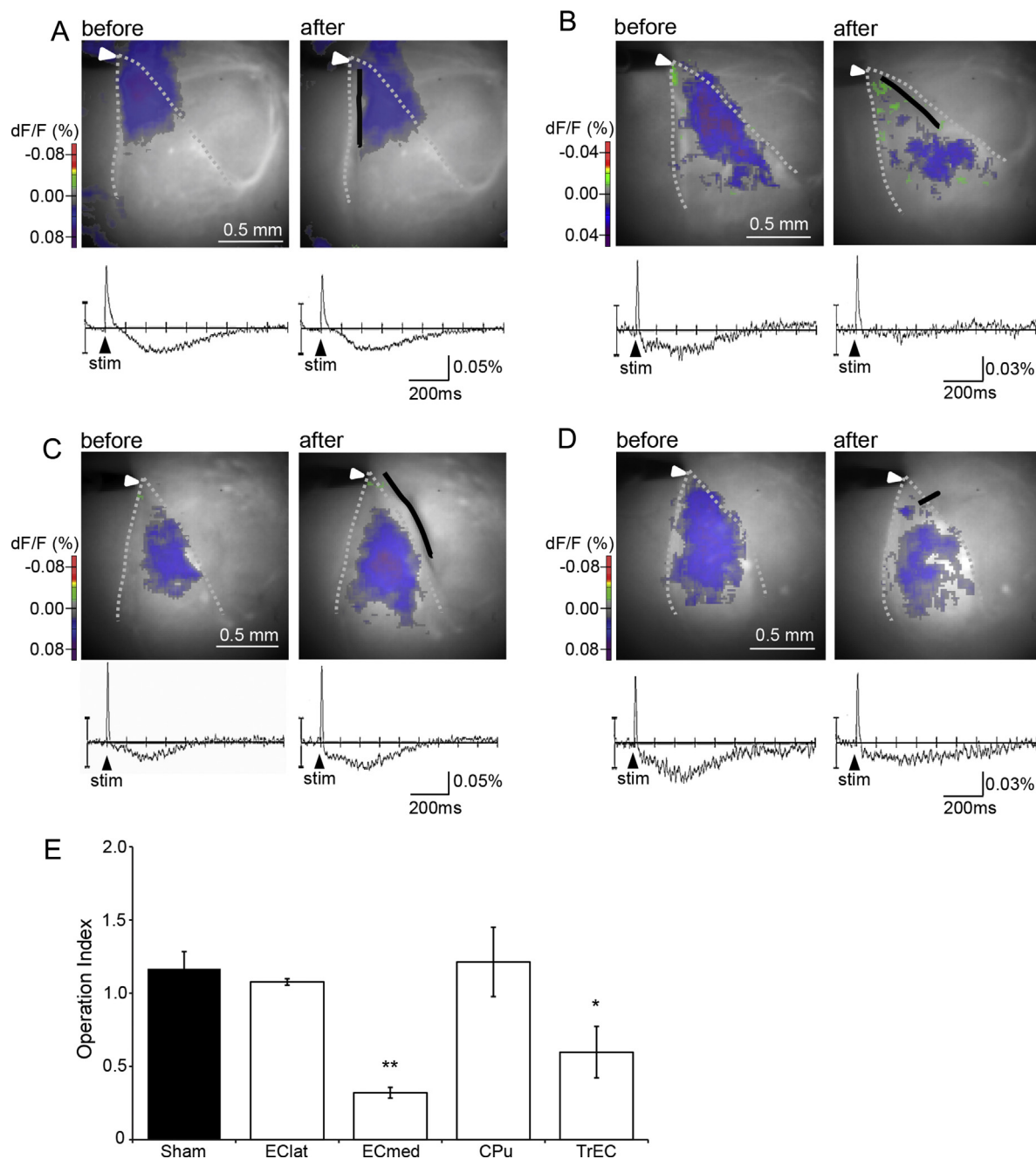
Next, to investigate the involvement of inputs from the main part of the CPu, we cut off the input from the CPu to the ECmed. In this experiment, the connections between the ECmed and the La remained. The OI values after deafferentation did not significantly change (Fig. 2C and E), suggesting that the inputs from the CPu are

not involved in the hyperpolarization induced in the La. For further investigation of the inputs via ECmed, a transverse cut at the dorsal part of the ECmed was carried out. As a result, the hyperpolarization was significantly reduced in the La (Fig. 2D). These data suggest that the fibers running along the ECmed were involved in the induction of hyperpolarization.

### 3.3. Effects of glutamatergic antagonists and the source of the inhibition

Finally, to confirm the details of the inhibitory input source and its properties, the effects of glutamatergic antagonists (6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10  $\mu$ M, TOCRIS), D-(–)-2-amino-5-phosphonopentanoic acid (D-AP5, 50  $\mu$ M, TOCRIS)) on the inhibitory responses were investigated. The inhibitory responses in the La induced by EC stimulation were statistically significantly reduced after the application of glutamatergic antagonists (Fig. 3A), suggesting that excitatory glutamatergic inputs were involved in the inhibitory response.

We then stimulated several parts along the ECmed in the presence of glutamatergic antagonists (Fig. 3B). Stimulation at the dorsal part of the ECmed did not induce the hyperpolarization (Fig. 3Ba); however, the hyperpolarization could be induced when the middle part of the ECmed was stimulated (Fig. 3Bb). When the electrode moved to the ventral part of the ECmed, weak hyperpolarizing responses were observed (Fig. 3Bc). The magnitude of the hyperpolarization induced by the stimulation at the middle part of the ECmed was statistically significantly larger than that induced by



**Fig. 2.** Effects of deafferentation of the lateral amygdala nucleus on the inhibitory responses.

(A–C) Afferent pathways to the lateral amygdala nucleus were surgically cut from the lateral branch of the external capsule (EClat) in (A), the medial branch (ECmed) in (B), and the caudate putamen (CPu) in (C) to the La was performed, respectively. Black lines, the surgical cut sites; white arrowhead, the stimulating site. The upper images of panels A, B, and C show the spread pattern of hyperpolarization before and after operation. Lower traces, the optical signal traces; black arrowhead, the timing of electrical stimulation. (D) The dorsal part of the ECmed was cut transversely (TrEC). (E) Operation indices (OIs) were quantitatively analyzed among various deafferentations. OIs of ECmed ( $n=6$  slices from 6 mice) and TrEC ( $n=7$  slices from 4 mice) were significantly lower than those of Sham ( $n=5$  slices from 3 mice), EClat ( $n=5$  slices from 4 mice), and CPu ( $n=7$  slices from 4 mice). \* $p<0.05$ ; \*\* $p<0.01$ .

the stimulation at the other parts (Fig. 3Bd). These results suggest that inhibitory neurons located in the middle part of the ECmed are the main source of the hyperpolarization in the La.

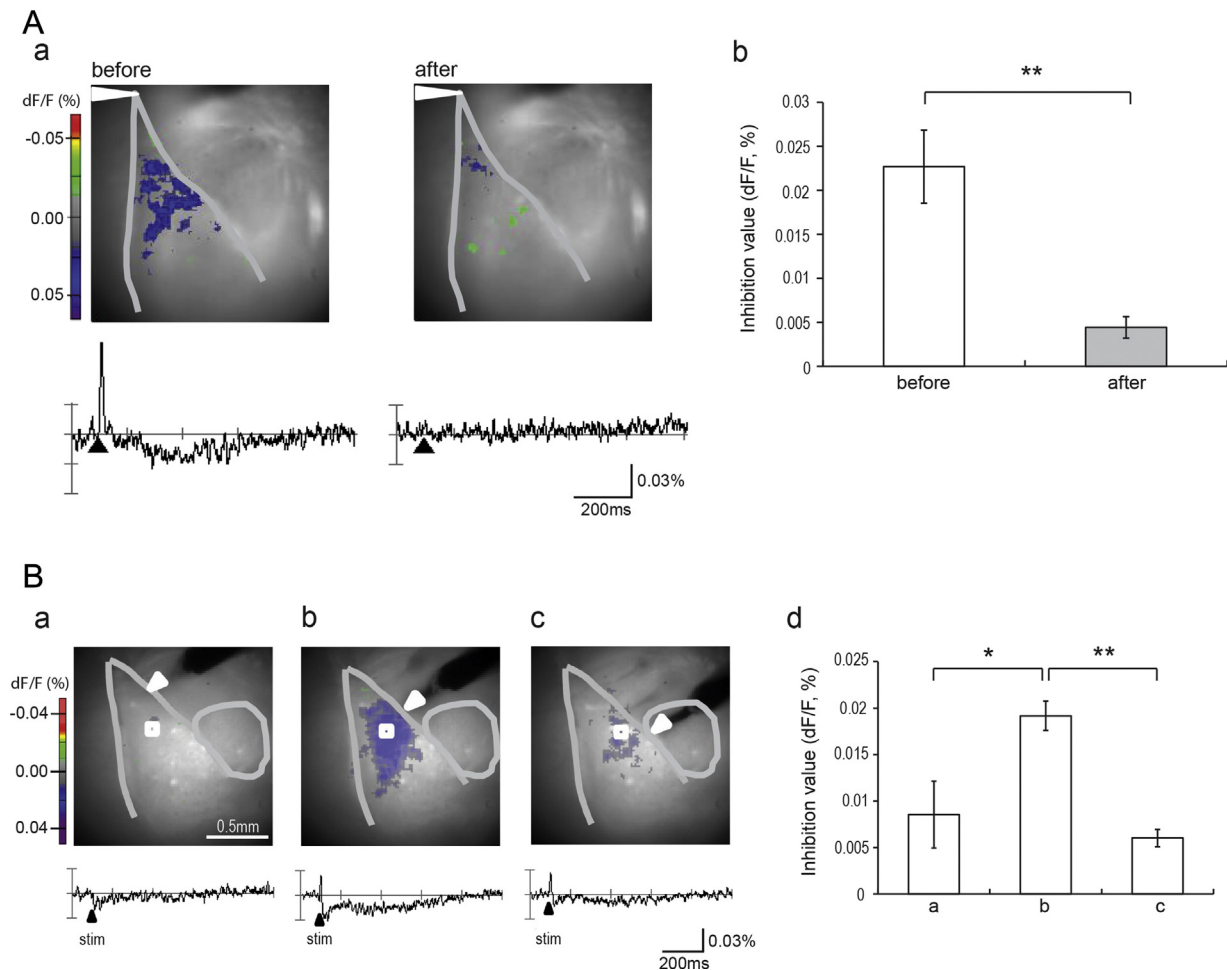
#### 4. Discussion

VSD imaging is a powerful tool to investigate spatial and temporal patterns in the propagation of membrane potential change in the brain tissue. Furthermore, optical signals from the stained slice preparation with VSD are reported to be well-correlated to field EPSPs in vitro [7,8,10,19,25,29,32]. In the amygdala, the

signal propagations from the La to the BLA [35,36] and from the La to the CeA have been visualized using VSD imaging [3]. However, these previous studies focused on the propagation of depolarizing signals in the amygdala but not of hyperpolarizing signals.

In the present study, we demonstrated the propagation of the inhibitory responses in the amygdala formation using VSD imaging. The strong and long-lasting hyperpolarization is evoked in the La after the sharp and strong depolarization. In addition, the surgical-cut and the pharmacological experiments indicate that the source of the inhibitory responses is not located in the La or the CPu, but is located in the middle part of the ECmed. According to this





**Fig. 3.** Effects of glutamatergic antagonists on the inhibitory responses.

(A) The effects of glutamatergic antagonists (CNQX + D-AP5) on the inhibitory response induced by the stimulation of the ramifying point of the external capsule. (Aa) Left and right upper images indicate the spread pattern of hyperpolarization before and after the drug application, respectively. White arrowhead, the stimulating site; lower traces, the optical signal traces; black arrowhead, the timing of electrical stimulation. (Ab) Inhibitory value was statistically compared between before and after CNQX + D-AP5 application ( $n = 5$  slices from 5 mice;  $**p < 0.01$ ). (B) The dependence of the stimulus position on inhibitory response of the La in the presence of CNQX + D-AP5. (Ba–c) Upper images indicate the spread pattern of hyperpolarization when the stimulus electrode was at the dorsal, middle, and ventral part of the ECmed, respectively. White arrowhead, the stimulating site; lower traces, the optical signal traces; white squares, the area of which the traces were calculated; black arrowhead, the timing of electrical stimulation. (Bd) Inhibitory value was shown when the stimulus electrode was at the dorsal (a), middle (b), and ventral (c) part of the ECmed ( $n = 4$  slices from 4 mice;  $*p < 0.05$ ;  $**p < 0.01$ ).

anatomical location, the source of the inhibitory inputs is likely from the m-ITC in the ECmed [5,18,20,27]. The inhibitory responses were completely eliminated in the presence of the glutamatergic antagonists, indicating that the inhibitory responses are based on the glutamatergic input passing through ECmed. Furthermore, local stimulation of the middle part of the ECmed produces inhibitory responses even in the presence of glutamatergic antagonists, suggesting that GABAergic neurons in the m-ITC are the source of the inhibitory responses of the La. A TrEC cut results in a smaller reduction of the inhibitory responses than an ECmed cut. Because it has been reported that stimulation of the La and the BLA excites the m-ITC neurons [27,28], excitatory glutamatergic inputs to the m-ITC through the La and the BLA might be involved in the inhibitory response in addition to the glutamatergic inputs coming along the ECmed.

In the present study, the contribution of the lateral intercalated clusters located in the EClat was smaller than that of the m-ITC, although the pathway from the lateral intercalated clusters to the BLA has been shown anatomically and electrophysiologically [16,30]. Because the direction of the stimulus electrode used in the present study was from the EClat to the ECmed, resulting in the

selective stimulation of the ECmed, the contribution of the lateral intercalated clusters to the inhibitory response in vivo cannot be excluded.

It has been reported that the stimulation of the La and the BLA excites the m-ITC neurons [27,28], resulting in the inhibition of the CeA neurons [2,27]. This pathway is suggested to be involved in the fear extinction mechanism [1], while the inhibitory inputs from m-ITC to La might interfere with the acquisition of fear memory.

In summary, the present study demonstrates the spatial and temporal spread pattern of the inhibitory response in the La after the stimulation of the EC using VSD imaging. Moreover, it is indicated that the inhibitory response is induced via the m-ITC. The pathway from the m-ITC to the La might have inhibitory effects on the acquisition of fear memory.

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